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The virulence of Bacillus anthracis has been attributed to a tripartite toxin composed of three proteins designated protective antigen (PA), lethal factor (LF), and edema factor (EF). The effects of the toxin components on human PMN phagocytosis and oxidative metabolism, as measured by chemiluminescence (CL), were studied in vitro. Initially, it was determined that the avirulent Sterne strain of B. anthracis (radiation killed) required opsonization with either serum complement or antibodies against Sterne cell wall in

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order to be phagocytized. Phagocytosis of the opsonized Sterne cells was not affected by the individual anthrax toxin components. However, a combination of PA and EF inhibited Sterne cell phagocytosis and blocked both particulate and soluble induced PMN CL. These PMN effects were reversibie upon removal of the toxin components. The PA and EF combination also increased intracellular cAMP levels. These studies suggest that two of the protein components of anthrax toxin, EF and PA increase host susceptibility to infection by suppressing PMN function and impairing host resistance.

ABSTRACT

The virulence of Bacillus anthracis has been attributed to a tripartite toxin composed of three proteins designated protective antigen (PA), lethal factor (LF), and edema factor (EF). The effects of the toxin components on human PMN phagocytosis and oxidative metabolism, as measured by chemiluminescence (CL), were studied in vitro. Initially, it was determined that the avirulent Sterne strain of B. anthracis (radiation killed) required opsonization with either serum complement or antibodies against Sterne cell wall in order to be phagocytized. Phagocytosis of the opsonized Sterne cells was not affected by the individual anthrax toxin components. However, a combination of PA and EF inhibited Sterne cell phagocytosis and blocked both particulate and soluble induced PMN CL. These PMN effects were reversible upon removal of the toxin components. The PA and EF combination also increased intracellular cAMP levels. These studies suggest that two of the protein components of anthrax toxin, EF and PA, increase host susceptibility to infection by suppressing PMN function and impairing host resistance.

# The Effects of Anthrax Toxin Components on Human Neutrophils

Infection and Immunity

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In conducting the research described in this report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals," as promulgated by the Committee on the Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council. The facilities are fully accredited by the American Association for Accreditation of Laboratory Animal Care.

The views of the authors do not purport to reflect the positions of the Department of the Army or the Department of Defense.

## Introduction

Keppie, et al. (1963) found that a crude mixture of three toxin components of Bacillus anthracis (protective antigen, edema factor, and lethal factor) decreases host resistance to an in vivo B. anthracis challenge and inhibits phagocytosis in vitro. The authors conclude that the toxins are virulence factors in B. anthracis pathogenesis. Until recently, there has been little research done to define these toxic effects.

Leppla (8) has recently reported the preparation of highly purified anthrax toxin factors and their effects on cultured eukaryotic cells. The availability of these toxin components presented us with an opportunity to reinvestigate the early work on their effects (7). Leppla (8) found edema factor (EF) to be an inactive adenylate cyclase, probably activated by eukaryotic cell calmodulin. In intact Chinese hamster ovary cells, protective antigen (PA) and EF cause a rapid increase of intracellular cAMP; PA is apparently required to facilitate EF entry into the cells (8). The adenylate cyclase activity of EF is potentially important to the virulence of B. anthracis; it has been reported (6,5) that an increase in the cAMP concentration in polymorphonuclear neutrophils (PMN) is associated with an inhibition of phagocytosis.

In the present study, the serum opsonic factors required for the phagocytosis of the avirulent Sterne strain of B. anthracis by human PMN were investigated. Also studied were the effects of the toxin components on PMN phagocytosis and oxidative metabolism, as measured by chemiluminescence (CL). A combination of PA and EF inhibited phagocytosis, blocked both particulate and soluble-induced PMN CL, and increased PMN intracellular cAMP levels.

## MATERIALS AND METHODS

**Leukocyte preparation.** All experiments were done with human PMN prepared from blood obtained via venapuncture of healthy volunteers. The cells were

isolated by dextran sedimentation and gradient centrifugation, as described (9), except the final cell stock was suspended at 1 x $10^6$ cells/ml in a modified barbital (Veronal) buffer ), henceforth called barbital buffer.

**Bacteria preparation.** B. anthracis (Sterne Strain) was grown in brain heart infusion (Difco) supplemented with 0.5% sodium bicarbonate in sealed screw-top flasks. Cultures were incubated at 37°C for 12 h. Colony-forming units (CFU) were determined via a 10 fold dilution in Hanks Balanced Salt Solution and culturing on trypticase soy agar, 37°C, overnight. The remainder of the culture was made to 5% with glycerol, distributed in 15 ml aliquots, placed on ice, and gamma-irradiated (3 x $10^6$ rads). Sterility was confirmed by plating an irradiated sample on blood agar. Sterile cells were stored at -70°C.

**Sterne cell wall preparation.** Frozen Sterne strain cells were thawed and plated on blood agar medium and inoculated into 1 liter of Casamino Acid medium (Difco), supplemented with 0.4% glucose. Following incubation (37°C, 18 h), the culture was transferred to a 20-liter fermentor containing the same medium. After 4 h incubation at 37°C with agitation at 150 rpm, dextrose was added to 0.4%, and incubation continued for 2 h. Cells were harvested via flow through centrifugation at 10,000 x g, resuspended in water at 0.2 g wet weight/ml, and sonicated (5/8 inch probe, 85% output) on ice for 45 min (nine 5 min bursts). Following centrifugation at 10,000 x g for 15 min the pellet was washed once with 40 ml water, and centrifuged at 27,000 x g for 10 min. The pellet was resuspended in 1% sodium dodecyl sulfate to 0.25 g wet weight/ml and sonicated for 1 min (5/8 inch probe, 50% output). The sample was heated to 85°C and centrifuged at 17,000 x g for 10 min. The supernatant was discarded, and the 1% sodium dodecyl sulfate treatment repeated twice. The pellet was washed three times with water (85°C). Following centrifugation

of each extraction, the dark material at the bottom of the cell pellet was discarded, and only the upper white material retained. The final cell wall material was lyophilized.

**Sera.** Antiserum to Sterne cell wall peptidoglycan material was obtained following weekly im vaccinations with 100 µg of material into a female New Zealand white rabbit. Titers were determined following heat treatment at 56°C, 30 min by microagglutination using Falcon microtiter plates in which diluted antisera (50 µl) were mixed with 50 µl sonicated Sterne cell wall material in saline (100 µg/ml). After overnight incubation, 4°C, titers were taken as being the last titration which produced agglutination. Aliquots of the serum were stored at -70°C.

Sera from non-immunized rabbits were collected and distributed. Some sera were stored at -70°C as a source of complement, while others were heat-treated at 56°C for 30 min for use as complement-free sera.

**Opsonization.** Aliquots (50 µl) of either Sterne cells or a boiled solution of zymosan (2 mg/ml, Sigma) in saline, were added to 2.0 ml of barbital buffer and centrifuged at 400 x g for 10 min. The supernatant was discarded leaving approximately 100 µl of buffer over the pellet. Sera to be used for opsonization were added (50 µl), and the tubes incubated for 30 min at 37°C. Treatment was ended by adding 2.0 ml barbital buffer and centrifuging the sample for 10 min at 400 x g. The resulting pellet was resuspended to 0.5 ml in barbital buffer, except for the zymosan, which was suspended to 5.0 ml.

**Chemiluminescence.** CL was determined at ambient temperature, under incandescent lighting, in a Beckman LS 6800 scintillation counter (Irvine, CA) using the Single Photon Monitor accessory. Assays were performed in plastic scintillation vials containing 1.8 ml of barbital buffer and 0.1 ml of 0.25 µM luminol. Luminol was stored as a 25 mM stock in dimethyl sulfoxide (DMSO) and

diluted with barbital buffer just prior to use. PMN suspension (20 µl) was added to each vial and CL intensity (CPM) determined by 0.1 min measurements at 20 min intervals. After 2 counting cycles to establish a uniform background luminescence, PMN CL was initated by adding 0.1 ml of opsonized bacteria or zymosan, or 25 µl phorbol myristate acetate (PMA). The PMA was stored as a 40 µM stock in DMSO. CL, measured at 20 min intervals, was determined for 120 min and all values corrected for background CL. In the case where there was a pre-treatment period with the anthrax toxin factors, CL was initiated at the end of that period by the addition of the CL inducers.

**cAMP assay.** For the assay of cAMP, human PMN from normal donors were collected by dextran sedimentation and Ficoll-Hypaque centrifugation. Residual RBC were lysed in $NH_4Cl$ buffer, and the PMN suspended in 1 ml RPMI 1640 with 10% serum at 1 x $10^7$ cells/ml. The PMN were incubated with PA and EF at 1 µg/ml each for 2 h at 37°C. After centrifugation and washing with HBSS twice, the cells were extracted with 0.2 ml 0.1 $NH_4Cl$ and cAMP measured by radioimmunoassay after acetylation (2) using an assay kit from New England Nuclear Corp. (Boston, MA).

**Microscopy.** At 50 minutes into the CL assay, PMN were subject to microscopic evaluation to determine if phagocytosis was occurring. One series of samples was concentrated by gentle centrifugation, (50 x g, 5 min) and observed under oil immersion phase contrast microscopy. In other samples phagocytosis was stopped after 1 h with the addition of paraformaldehyde to a final concentration of 2%. Cells were pelleted, fixed in Karnovsky's fixative (K-1965) for 1 hour at room temperature, embedded in agar, and postfixed 1 h in 1% $OsO_4$. Pellets were then incubated in 0.5% uranyl acetate, dehydrated through a graded ethanol series, and embedded in Epon 812. Thin sections,

stained with uranyl acetate and lead citrate, were observed with a JEOL JEM 100B electron microscope at 80 kV.

**Anthrax toxin components.** The anthrax toxin components were prepared as described previously (8). Aliquots of each factor (1 mg/ml in 10 mM HEPES, 2 mM B-merceptoethanol) were stored at -70°C. Prior to use, aliquots were rapidly thawed, diluted in barbital buffer, and supplemented with 0.1% bovine serum albumin. The stock preparations were not re-frozen, but kept for 3 weeks on ice. After that time, activity decreased and fresh stocks were prepared.

## RESULTS

Our initial experiments characterized the particulate induced CL response of human PMN exposed to the avirulent Sterne strain of B. anthracis. Sterne cells treated with heat-inactivated non-immune sera were unable to induce a CL response in human PMN and served as the control for background CL. Bacterial cells treated with either fresh non-immune sera (complement opsonized) or heat treated immune sera (antibody opsonized) induced dose and time dependent increases in PMN CL (Fig. 1).

The highest CL response seen in Fig. 1 represents the maximum response of the PMN since it was not increased by adding 4 fold more bacteria or using 4 fold more sera. The importance of establishing these maximum response conditions in studying phagocytosis has been discussed (11). The radiation-killed Sterne preparation, whose optimal concentration was determined as shown in Fig. 1, was used in all subsequent experiments at a CFU to PMN ratio of approximately 30:1. The preparation gave repeatable results for 1 year when stored in aliquots at -70°C. Other lots of Sterne have since been used with

results similar to those reported here, although each lot had to be separately characterized for the optimal CFU to PMN ratio.

In order to determine the relationship between induction of CL and phagocytosis, samples were collected directly from the scintillation vials at the height of the CL response. The PMN controls, in which CL was not being induced, were found under phase contrast microscopy (1000 x) to be rounded and unassociated with bacilli (Fig. 2A). However, the PMN exposed to complement opsonized Sterne cells had an irregular, distorted shape, and were associated with multiple bacilli (Fig. 2B). Electron microscopic studies on the same samples revealed internalized bacilli (Fig. 2C), while the controls were negative.

The effects of the three anthrax toxin components were tested separately and in combination with respect to their effect on the induction of PMN CL using complement-opsonized Sterne cells. If bacteria and toxin component(s) were added simultaneously, a normal CL response, similar to that shown in Fig. 1, was obtained. If, however, the opsonized Sterne were added 60 min after the addition of toxin components, a mixture of PA and EF dramatically supressed CL (Fig. 3). Inhibition was not found with any of the toxin components individually or in other combinations. Although not shown, LF was without effect on the PA and EF inhibition of CL when all 3 components were at 1 µg/ml.

The PA and EF effect was maximal following a 60 min pretreatment with the toxin combination. Studies with phase contrast microscopy showed that the PA and EF treated PMN, which had shown suppressed CL response to opsonized Sterne bacilli, were spherical and unassociated with bacteria, indicating that phagocytosis was inhibited. In addition, we found that, following a 60 min exposure of PMN to PA and EF, if the cells were re-suspended for an additional h in

buffer without toxins, a normal CL response was induced by opsonized Sterne cells. Thus the effects of the toxin components were readily reversed.

The effect of the PA and EF combination on the PMN CL response to inducers other than complement opsonified Sterne cells was determined. Suppression of CL induction was found when PMN were exposed to either Sterne cells opsonified with antibody or to the soluble CL inducer PMA (Fig 4). Complement opsonized zymosan gave similar results (data not shown).

Since EF has been reported to be an adenylate cyclase (8), studies were designed to determine the effect of PA and EF on PMN cAMP concentrations. PA together with EF caused a significant increase in intracellular cAMP in PMN from a control value of $3.7 \pm 0.2$ pmol/$10^7$ cells to $23.2 \pm 0.8$ pmol/$10^7$ cells. The increase varied from 2 to 13 fold in 3 separate experiments using 3 different donors. There was no effect when either factor was added individually.

## DISCUSSION

The Sterne strain of B. anthracis is used as a live vaccine in domestic animals. Its avirulence has been attributed to the absence of the anti-phagocytic capsule found on fully virulent strains (7,12). It has been reported that the opsonized virulent Vollum strain of B. anthracis will not induce a CL response in human neutrophils, irrespective of the opsonins used (9). Results reported here are the first to demonstrate that the opsonized Sterne strain is phagocytized, and that opsonization is readily accomplished with either complement or Sterne strain cell wall antibodies.

The observation of Keppie et al. (7), upon which these studies were based, is that a mixture of the 3 toxin components of B. anthracis inhibited

guinea pig PMN phagocytosis in vitro. Our studies have futher defined that effect using highly purified toxin components, and have found that the anti-phagocytic activity is due to a combination of PA and EF, and is associated with an increase in cellular cAMP.

The inhibitory effect of the PA and EF combination on the induction of PMN CL cannot be attributed to quenching, since the CL response is unaffected when the toxin components are added simultaneously with the CL inducer. The effects of the toxin combination appear to be at the cellular level. While little is known of the mechanism of phagocytic-induced PMN CL, it is obvious that if phagocytosis is inhibited, there will be no phagocytic induced CL response. It is not evident, however, why the PMA, or non-phagocytic induction of CL is inhibited by the toxin combinations. While PMA and the particulate inducers of CL have similarities (4), they do involve different mechanisms. This is best demonstrated by the observation that while cytochalasin B blocks both zymosan-induced phagocytosis and the corresponding CL response, it does not inhibit PMA induced CL (14). At this time we do not know if PA and EF inhibit phagocytosis and CL separately or on a common pathway. It is also possible that the block of PMA-induced CL is due to inhibition of uptake of PMA since PA and EF inhibit pinocytosis in macrophages (A. Friedlander, unpublished observations).

In a previous report, EF, in the presence of PA, was shown to act as an adenylate cyclase in Chinese hamster ovary cells (8). It has been proposed that the stimulation of intracellular PMN cAMP leads to an inhibition of phagocytosis (6,5). An exception is the effect of cholera toxin which has been shown to augment cAMP levels in PMN, yet does not inhibit phagocytosis (1). It has been reported that Bordetella pertussis produces an adenylate cyclase that decreases PMN bactericidal activity (3). Here we report that PA

and EF increase PMN cAMP, which coincides with phagocytic inhibition. In view of the disparities between the effects of cholera toxin and other augmenters of cAMP, it is clear that the exact relationship between phagocytosis, cAMP levels, and CL remain to be determined. While fatal anthrax is associated with supressed host immune defense (7,12), little is known concerning the in vivo production and involvement of the anthrax toxin proteins (13). These studies suggest, however, that EF in the presence of PA may increase host susceptibility to infection by suppressing PMN function.

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FIG. 1. CL intensity versus time of human PMN exposed to different concentrations of Sterne strain of B. anthracis opsonized with either complement (A) or antibody (B). The CFU to PMN ratio was 30:1 (o), 15:1 (●), 7.5:1 (Δ) and 1:1 (▲) Each data point is the mean ± SEM from 5 separate experiments, each experiment using PMN from a different individual.

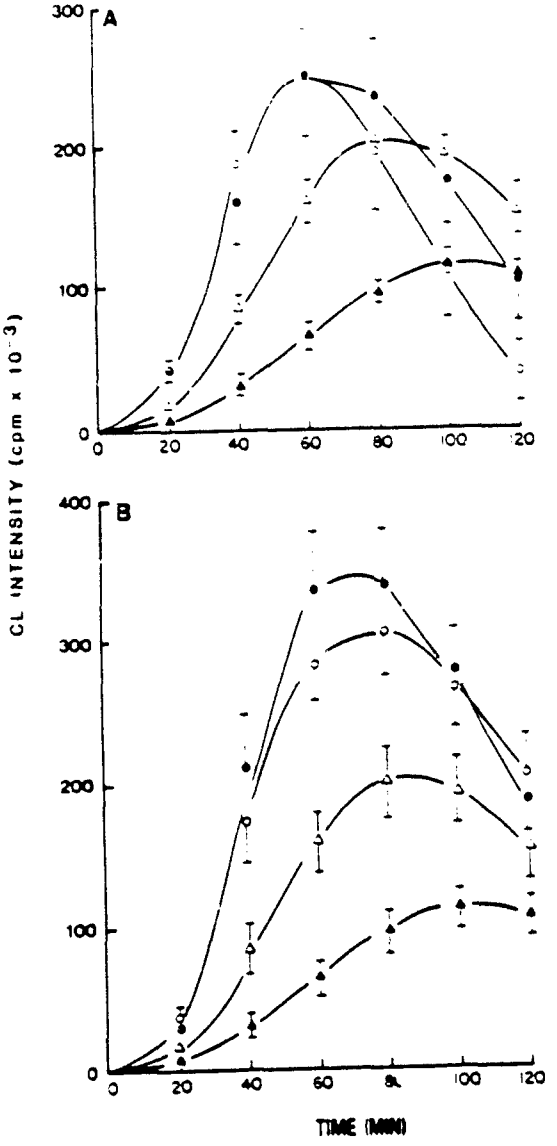

A
B
CL INTENSITY (cpm x 10⁻³)
TIME (MIN)
0
100
200
300
400
20
40
60
80
120

FIG. 2. Microscopic studies of phagocytosis of Sterne strain of B. anthracis by human PMN. A. Control PMN, under phase microscopy, exposed to bacteria treated with heat-inactivated non-immune serum (no opsonins), which showed no stimulation of CL activity. B. Phase microscopy of PMN exposed to bacteria opsonized with complement, at the time of maximum CL induction. C. Electron micrograph of PMN from the same sample as shown in Fig. B. Arrows denote internalized bacilli. The assay conditions were as in FIG. 1. The CFU to PMN ratio was 30.

Fig 2

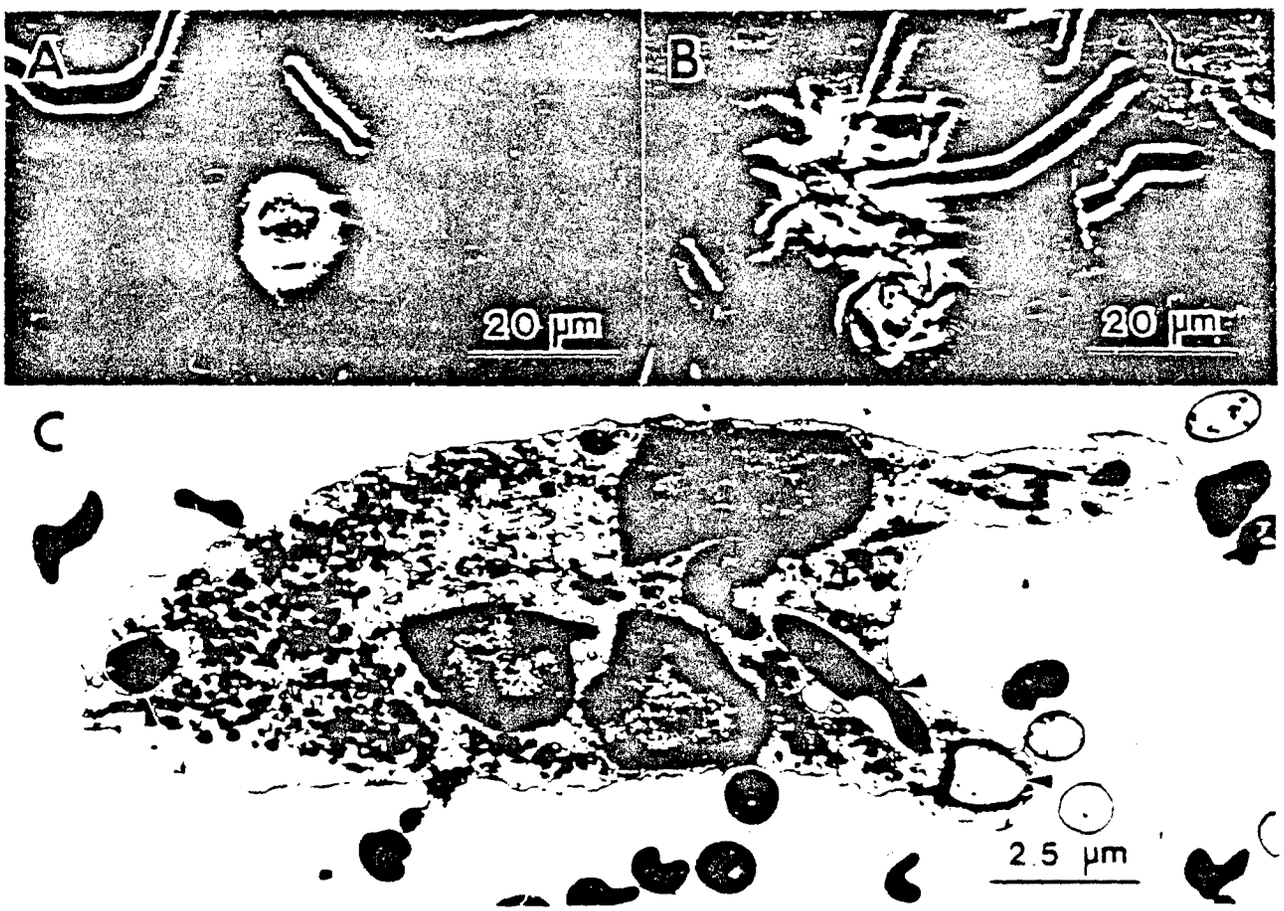


FIG. 3. Effect of anthrax toxin components on CL induced in human PMN with complement opsonized Sterne strain of B. anthracis. The toxin components (1 µg/ml) were introduced to the PMN 1 h prior to the addition of the bacteria. The CL response is reported as the maximum CL intensity (CPM) ± SEM on triplicate determinations. Assay conditions were as in FIG. 1. The CFU to PMN ratio was 30.

Fig 3

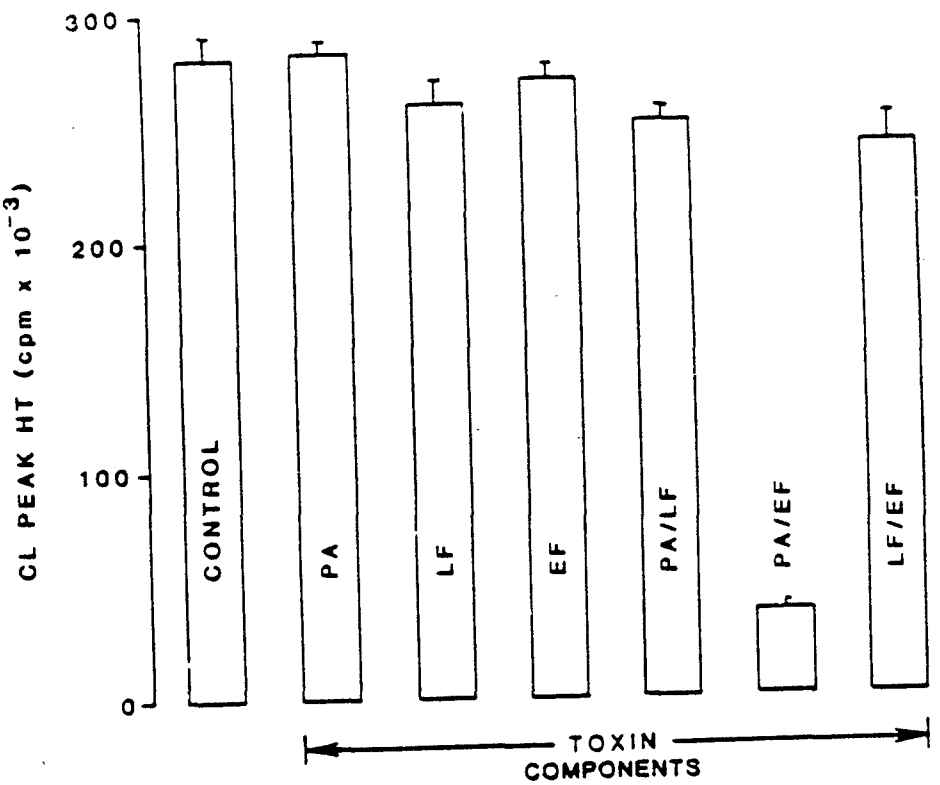
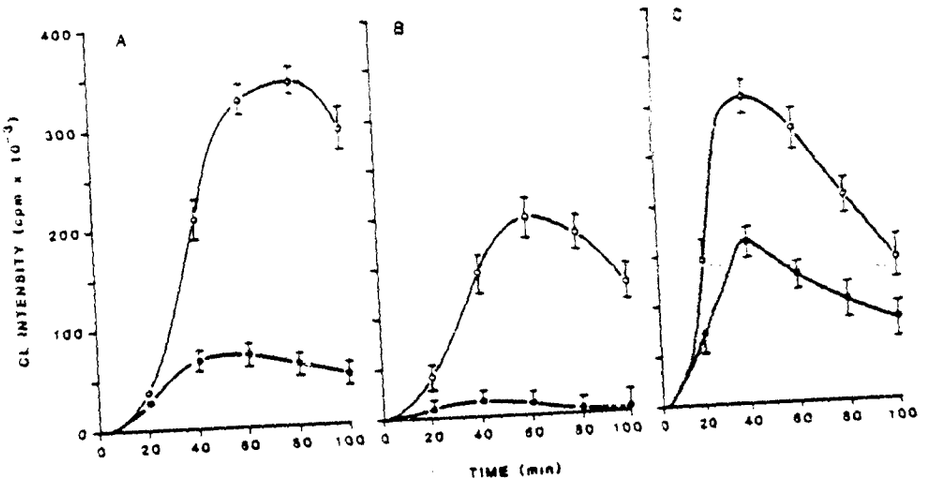

300
200
100
0
CL PEAK HT (cpm x $10^{-3}$)
CONTROL
PA
LF
EF
PA/LF
PA/EF
LF/EF
TOXIN
COMPONENTS

FIG. 4. Effect of PA and EF on the stimulation of human PMN CL using different inducers. PMN were pretreated with the toxin components for 1 h prior to addition of the CL inducer. Induction of CL was with antibody opsonized Sterne (A), complement-opsonized Sterne (B), and PMA (C). Samples without toxin are indicated by (o) and with toxin by (•). Data points are means of triplicate determinations ± SEM. Assay conditions were as in FIG. 1. The CFU to PMN ratio was 30.

Fig 4


A
B
C
CL INTENSITY (cpm x $10^{-3}$)
400
300
200
100
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0
20
40
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80
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TIME (min)